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# Evaluation of Interallelic *waxy*, Hetero*waxy*, and Wild-Type Grain Sorghum Hybrids

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RESEARCH

# Evaluation of Interallelic *waxy*, Hetero*waxy*, and Wild-Type Grain Sorghum Hybrids

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## ABSTRACT

Four near-isogenic Wheatland × Tx430 grain sorghum [*Sorghum bicolor* (L.) Moench] hybrids differing in allelic status at the *Waxy* locus were grown in yield trials to determine their potential to expand existing sources of low-amylose starch. The hypothesis tested was that agronomic performance and grain yield do not differ among hybrid genotypes. Hybrids were generated in a two-by-two factorial design using $wx^b$ and wild-type (WT) Wheatland as female parents with $wx^a$ and WT Tx430 as male parents. Yield trials were conducted at two Nebraska locations in 2009 and 2010. No differences were observed for field emergence, but grain yield of the interallelic *waxy* ($wx^b \times wx^a$) hybrid was 330 kg ha$^{-1}$ greater than the WT × WT hybrid ($P$ = 0.0482). The $wx^b \times Wx$ hybrid had the highest grain yield, 633 kg ha$^{-1}$ greater than the WT ($P$ = 0.0003). Amylose starch content was lowest for $wx^b \times wx^a$ (7.66 g kg$^{-1}$), intermediate for $wx^b \times Wx$ and $Wx \times wx^a$ (25.06 and 27.20 g kg$^{-1}$, respectively); and highest for WT × WT (34.80 g kg$^{-1}$) ($n$ = 4, $P$ < 0.0001). The *waxy* and hetero*waxy* hybrids evaluated in this study are promising options for commercial production of starches with reduced amylose contents in a drought-tolerant crop.


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**Abbreviations:** DSC, differential scanning calorimetry; FAN, free amino-N; GBSS, granule-bound starch synthase; HD, highly digestible protein; LPC, 1-α-lysophosphatidylcholine; TDN, total digestible nutrients; WT, wild-type.

Starch is made up of two types of polymers: amylose and amylopectin. Amylose (106–108 kDa) is relatively linear, consisting of α-1,4-linked glucose units with α-1,6-linkage branch points every 200 to 10,000 units (Tester et al., 2004; Tetlow 2011). Amylopectin (108–109 kDa) is more highly branched than amylose with α-1,6-linkage branch points occurring approximately every 20 glucose units (Tetlow 2011). Branching in amylopectin occurs in a regular pattern, resulting in alternating concentric crystalline and amorphous lamellae (Hizukuri 1986; Jenkins et al., 1993; Perez and Bertoft 2010). The physical configurations and ratio of amylose and amylopectin in starch are key factors in determining its function as a food ingredient and its efficiency as a biofeedstock. Amylose forms complexes with lipids and increases starch viscosity during cooking (Copeland et al., 2009; Jobling 2004), reducing its digestibility for animals (Liu et al., 2014; Zhu et al., 2011), and in fermentation systems (Li et al., 2014; Wu et al., 2007, 2010; Yan et al., 2011). Amylopectin does not complex with lipids and exhibits reduced viscosity and retrogradation, thus improving the shelf life of breads, cakes, and pastes (Wang and Copeland 2013) but also increasing the glycemic index of cooked foods (Behall and Hallfrisch 2002). Its improved digestibility substantially increases fermentation efficiency into ethanol (Wu et al., 2007).

The amylose/amylopectin ratio of starch (generally 30:70 in cereal endosperms) is primarily under genetic control. The *Waxy* (*Wx*) gene encodes the granule-bound starch synthase (GBSS)

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enzyme, which synthesizes amylose within starch granules (Denyer et al., 2001). Loss-of-function mutations in *Wx* result in an endosperm with a waxy appearance and starch almost entirely composed of amylopectin (Denyer et al., 2001; Nelson and Rines 1962). Predominant commercial sources of low-amylose starch include *waxy* (*wx*) varieties or hybrids of maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and potato (*Solanum tuberosum* L.) (Swinkels 1985). Sorghum would be an ideal source of low-amylose starch, as it is more drought-tolerant than these crops (FAO, 1986). Thus, an opportunity exists to expand the supply of low-amylose starch through deployment of *waxy* sorghum hybrids.

The *Wx* gene displays simple Mendelian inheritance (Karper 1933), but amylose content of sorghum grain depends on the dose of *wx* alleles in the triploid endosperm (Ring et al., 1989). Hetero*waxy* $F_1$ plants resulting from WT × *wx* or *wx* × WT crosses produce $F_2$ seed segregating 1:1:1:1 for *WxWxWx*, *WxWxwx*, *Wxwxwx*, and *wxwxwx* endosperm genotypes, which, when pooled, have intermediate levels of amylose starch (approximately 20–25 g $kg^{-1}$) (Ring et al., 1989; Sang et al., 2008). Ethanol fermentation efficiency (Wang et al., 2008; Wu et al., 2010; Yan et al., 2011) and cooking and pasting properties (Sang et al., 2008; Zhu 2014) generally improve as amylose content decreases such that both *waxy* and hetero*waxy* grain have potential utility as biofuel feedstocks and as custom starches for the food industry.

Four mutations conferring the *waxy* phenotype have been identified in the sorghum *Wx* gene (*Sb10 g002140*) (Sattler et al., 2009; Kawahigashi et al., 2013; Lu et al., 2013). Previous research characterized two *wx* alleles for the presence or absence of GBSS protein in sorghum grain (Pedersen et al., 2005). The $wx^a$ allele contained a large DNA insertion within the third exon of WT *Wx*, and both the GBSS protein and enzyme activity were undetectable in $wx^a$ grain (Sattler et al., 2009). The $wx^b$ allele contained a missense mutation that changed amino acid 268 from a glutamine to a histidine that reduced enzyme activity by over 75% relative to the WT but did not affect the GBSS protein levels in the grain (Sattler et al., 2009). It is currently unknown whether $wx^a$ and $wx^b$ starches differ in amylose content.

Interest in grain sorghum has increased since 2012 when the USEPA announced that ethanol produced from sorghum grain qualifies as an advanced biofuel (USEPA, 2012). The ethanol fermentation efficiency benefits of *waxy* sorghum grain are well documented (Sharma et al., 2007; Wang et al., 2008; Yan et al., 2011; Wu et al., 2013). Despite the utility of *waxy* sorghum grain in the ethanol and food industries, the *waxy* trait has historically been associated with yield drag (Rooney et al., 2005), because of uncharacterized factors (Jampala et al., 2012; Yerka et al., 2015a), or its presence in unadapted genetic backgrounds. Further development and characterization of high-yielding *waxy* parent lines and hybrids should contribute to the commercialization of *waxy* sorghum for end uses in the ethanol and food industries. We previously introgressed $wx^a$ and $wx^b$ alleles into the grain sorghum lines A/B Wheatland and RTx430, respectively (see Materials and Methods section). The objective of this study was to evaluate the agronomic performance, grain yield, and amylose content of near-isogenic Wheatland × Tx430 hybrids with *waxy*, hetero*waxy*, or WT endosperm.

## MATERIALS AND METHODS

### Hybrid Production and Evaluation

The parent lines $wx^b$ Wheatland and $wx^a$ Tx430 were developed jointly by USDA–ARS and the University of Nebraska–Lincoln at the Agricultural Research and Development Center near Mead, NE (Yerka et al., 2015b). The $wx^b$ Wheatland seed parent (A/B heterotic group) line was generated through introgression of the $wx^b$ allele from BTxAGR-1 (Pedersen et al., 2007) followed by four generations of backcrossing to WT BWheatland (Brown et al., 1936) and subsequent sterilization in $A_1$ cytoplasm. The $wx^a$ Tx430 pollen parent (R heterotic group) line was generated through introgression of the $wx^a$ allele from RTx2907 (Pedersen et al., 2007) followed by four generations of backcrossing to WT RTx430 (Miller 1984). Hybrids were produced using WT AWheatland and $wx^b$ AWheatland as females in a two-by-two factorial design with WT RTx430 and $wx^a$ RTx430 serving as males. Near-isogenic $wx^b$ female parents and $wx^a$ male parents were not available for this study and were not included. The $F_1$ hybrids evaluated in yield trials were WT × WT (*Wx* × *Wx*), *waxy* ($wx^b$ × $wx^a$), hetero$waxy^b$ ($wx^b$ × WT), and hetero$waxy^a$ (WT × $wx^a$). Hybrid seed was sown at 120 seeds per row (240,000 seeds $ha^{-1}$) delivered by a precision vacuum planter in plots consisting of four 6.6-m rows spaced 76-cm apart. Trials were planted in the last week of May 2009 and 2010 at Mead, NE, and at Lincoln, NE, in a randomized complete block with five replicates. At Mead in 2009, 112 kg $ha^{-1}$ N fertilizer was applied preplanting. Atrazine [6-chloro-*n*-ethyl-*N*'(1-methylethyl)-1,3,5-triazine-2,4,diamine] was applied at 1.12 kg $ha^{-1}$ postplanting. Quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) and atrazine at 0.37 and 0.56 kg $ha^{-1}$, respectively, were applied approximately 3 wk postemergence. Supplemental irrigation (3.8 cm) was applied via overhead sprinklers on 6 and 29 August. Similar cultural methods were employed in 2010, except supplemental irrigation (3.8 cm) was applied 9 August. Similar cultural methods were employed at Lincoln in 2009 and 2010, except no postemergence chemical application was made in 2010 and no supplemental irrigation was provided in either year.

Field emergence (plants per meter) was recorded 4 wk after planting. Days to 50% anthesis and height (centimeters to the top of the mature panicle) were recorded. Nylon netting was applied postanthesis at Mead to reduce predation by birds. Grain was hand-harvested from the middle two rows of each plot, dried at 32°C to constant mass, weighed, and adjusted to 145 g $L^{-1}$ moisture before analysis. Grain yield (kg $ha^{-1}$) and grain volume weight (kg $L^{-1}$) were determined.

## Grain Composition

The grain of each hybrid was assessed for moisture, crude protein (Padmore 1990b), crude fiber, fat (Padmore 1990a), ash, starch (Hall 2001), and total digestible nutrients (TDN) by Ward Laboratories, Inc. (Kearney, NE) in units of gram per kilogram. Grain composition characters were assessed on a dry-weight basis.

Free amino-N (FAN) content (mg $L^{-1}$) of hybrid grain was determined as previously described (Wu et al., 2010). A FAN assay was conducted on a composite sample of grain from each hybrid ($n$ = 4) within each location ($n$ = 2) made by pooling across replicates and years within location (eight composite samples total). Grain was milled into flour with a Udy Cyclone sample mill to pass through a 1-mm screen (Udy Corp.). Three subsamples (150 mg) constituting three technical replicates of each composite sample, hereafter referred to as samples, were extracted in water (Yan et al., 2011). A ninhydrin assay was conducted on the resulting supernatant (500 μL) to determine FAN content (mg $L^{-1}$) according to the methods of Spedding et al. (2012) with modification. Ninhydrin (200 μL of 35:100 ninhydrin/ethanol [mg $L^{-1}$] solution) (Hwang and Ederer, 1975) was added to the supernatant and mixed by inverting three times. Samples were heated to 90°C for 10 min with lid locks to prevent evaporation and inverted three times during heating. Samples were quickly cooled to 20°C in a cold water bath. Aliquots (20 μL) of each sample were transferred to a microplate, diluted 1:5 (v/v) with a dilution solution (1 g potassium iodate [$KIO_3$] dissolved in 300 mL distilled water and 200 mL 95% ethanol) and analyzed for absorbance at 570 nm ($A_{570}$) on a Synergy HT microplate reader (BioTek Intstruments, Inc.). A glycine solution (exactly 26.8 mg glycine dissolved in 25 mL distilled water to contain 200 mg $L^{-1}$ FAN) was diluted between 0 and 100% with distilled water and subjected to the FAN assay concurrently with the samples to generate a standard curve (Spedding et al., 2012). The FAN assay was conducted twice.

Amylose and amylopectin gelatinization parameters and calculated amylose starch content were determined using the unmilled, composited samples used in the FAN assay. Composite samples were divided into three subsamples (5 g) to constitute three technical replicates. Starch extraction and purification proceeded according to the methods of Pedersen et al. (2007). Purified sorghum starch samples and five wheat starch samples (0.10–0.80 g $kg^{-1}$ amylose) were uniformly dried in a desiccation chamber and then analyzed by differential scanning calorimetry (DSC) on a Pyris 7 Diamond DSC (PerkinElmer, Inc.). The three subsamples for each hybrid were analyzed in three separate runs on the DSC and wheat samples were included in each run. The DSC analysis proceeded according to the method of Hansen et al. (2010) except that samples (10–12 mg) were hydrated in 50 μL of 2% aqueous 1-α-lysophosphatidylcholine (LPC) solution (Polaske et al., 2005) and allowed to equilibrate at 20°C for 16 h. Samples were held at 25°C for 2 min, heated from 25 to 120°C at a rate of 10°C $min^{-1}$, held at 120°C for 2 min, and cooled from 120 to 30°C at a rate of 10°C $min^{-1}$. Heat flow (mW) was plotted against temperature to reveal three peaks corresponding to amylopectin and amylose gelatinization during the initial heating (endothermic) phase and formation of a complex between gelatinized amylose and LPC during the subsequent cooling (exothermic) phase. PyrisTM software was used to identify the onset, maximum, and end temperatures of each peak, peak area, and enthalpy of transition ($\Delta H$) (Sievert and Holm, 1993). Enthalpy associated with formation of the amylose–LPC complex was related to amylose content (Zhu and Jackson 2000) using the wheat starch samples as a standard curve.

## Statistical Analysis

Field and grain composition data were fit to a mixed model using PROC MIXED of SAS version 9.3 (SAS Institute, 2012). Environment, the interaction of environment with hybrid, and the replicates within environment were random effects in the model. Normality of residuals was assessed in PROC UNIVARIATE with a Shapiro–Wilke Test and by analysis of quantile–quantile plots. Homogeneity of variance was assessed with a Levene's Test in PROC GLM. When a significant $F$-test was observed, means were separated at $P$ = 0.05 using the PDIFF option with the LSMEANS statement.

Because of the use of composite samples in FAN and DSC analysis, these data were fitted to a linear mixed model using PROC GLIMMIX. Environment, the environment × hybrid interaction, and the replicates within environment were random effects in the model. A heterogeneous variance structure was fitted to the residual variance using the _RESIDUAL_ and GROUP options with the RANDOM statement. Homogeneity of the residual variance among hybrids was tested using the HOMOGENEITY option with the COVTEST statement. If the test had $P > 0.05$, the model was refit without the heterogeneous variance structure. The effect of hybrid was tested with an $F$-test from the chosen fitted model. When a significant $F$-test was observed, means were separated at $P = 0.05$ using the LINES option with the LSMEANS statement.

# RESULTS AND DISCUSSION

## Agronomic Performance

Mean squares from analysis of variance and least-squares means for agronomic performance data are shown in Tables 1 and 2, respectively. The majority of variance for all characters was explained by the environment (Table 1), although the effect of hybrid entry on height and grain yield was significant ($P < 0.05$), and the test hypothesis that no differences would be observed was rejected. Hybrid entry had no effect on days to 50% anthesis or on field emergence and grain volume weight due to altered grain composition when amylose content was significantly reduced in the $wx^b \times wx^a$ hybrid. Hybrid height differed ($n$ = 4, $P < 0.0001$) and correlated with $wx$ in a dose-dependent manner, with WT × WT being shortest (140 cm) and $wx^b \times wx^a$ being tallest (151 cm). Grain yield differed ($n$ = 4, $P = 0.0030$) and was highest for hybrids with the $wx^b$ Wheatland parent (9647 kg $ha^{-1}$ [$wx^b \times wx^a$] and 9949 kg $ha^{-1}$ [$wx^b \times$ WT]) compared with hybrids with the WT Wheatland parent (9539 kg $ha^{-1}$ [WT × $wx^a$] and 9325 kg $ha^{-1}$ [WT × WT]). Near-isogenic $wx^a$ female parents and $wx^b$ male parents were not available for this

**Table 1. Mean squares from the combined analysis of variance for agronomic performance of near-isogenic wild-type (WT), *waxy*, and hetero*waxy* Wheatland × Tx430 grain sorghum hybrids grown at four Nebraska environments: Mead and Lincoln, NE, in 2009 and 2010.**

| Source of variation | Df | Emergence | 50% anthesis | Height | Grain yield | Grain volume weight |
|---|---|---|---|---|---|---|
| | | plants $m^{-1}$ | d | cm | kg $ha^{-1}$ | kg $hL^{-1}$ |
| Hybrid (*H*) | 3 | 1.35 | 4.33 | 477.81** | 1,130,998* | 0.81 |
| Environment (*E*) | 3 | 34.68 | 1337.23** | 2851.15** | 26,310,168** | 10.61** |
| Rep (environment) | 16 | 6.51 | 11.24** | 16.41* | 185,594 | 0.70 |
| *E* × *H* | 9 | 8.92 | 2.63 | 15.03 | 206,311 | 0.40 |
| Residual | 46 | 5.63 | 1.58 | 8.07 | 267,727 | 0.61 |

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

**Table 2. Agronomic performance of near-isogenic wild-type (WT), *waxy*, and hetero*waxy* Wheatland × Tx430 grain sorghum hybrids. Data were pooled over four environments: Mead and Lincoln, NE, in 2009 and 2010.**

| Hybrid entry† | Emergence | 50% anthesis | Height | Yield | Grain volume weight |
|---|---|---|---|---|---|
| | plants $m^{-1}$ | d | cm | kg $ha^{-1}$ | kg $hL^{-1}$ |
| WT × WT | 14a‡ | 72a | 140a | 9317a | 71a |
| WT × $wx^a$ | 14a | 73a | 143b | 9539ab | 71a |
| $wx^b$ × $wx^a$ | 14a | 73a | 151d | 9647bc | 72a |
| $wx^b$ × WT | 14a | 73a | 147c | 9950c | 72a |

† $wx^a$ and $wx^b$ denote two recessive alleles conferring the *waxy* phenotype.

‡ Means followed by the same letter within columns do not differ at $P = 0.05$.

study; hence, comparison of yield among homozygous $wx^a$ and $wx^b$ hybrids relative to the interallelic *waxy* ($wx^b$ × $wx^a$) and WT hybrids is a focus of future research. In addition, Stam and Zeven (1981) showed that near-isogenic lines of self-pollinating plants developed through backcrossing are not completely isogenic because of tight linkage in some parts of the donor parent genome, and linkage drag could affect the trait of interest. Nevertheless, creation of the $wx^b$ × $wx^a$ hybrid was a strategy to overcome any potential yield drag associated with recessive deleterious linked genes at the *Wx* locus. Results demonstrate that the *waxy* and hetero*waxy* hybrids evaluated were not associated with a yield penalty relative to the WT hybrid.

Improving agronomic performance of *wx* lines and hybrids relative to WT has been difficult to achieve consistently (Jampala et al., 2012; Rooney et al., 2005; Yerka et al., 2015a). In particular, Yerka et al. (2015a) evaluated 11 pairs of grain sorghum lines near-isogenic for *wx* or WT: two *wx* lines (BN619 and BN635) differed in floral synchrony from their respective WT lines, and five *wx* lines (BN619, BN621, BN635, RN637, and RN639) differed in height. However, *wx* genotypes were not consistently associated with increased time to anthesis or increased height at maturity. Five *wx* lines (BN619, BN621, BN623, BN625, and BN635) produced less grain relative to respective WT lines, and one *wx* line (RN637) produced more grain. The combined grain yield of $wx^a$ lines did not differ from that of their near-isogenic WT counterparts, but combined $wx^b$ grain yield was reduced ($n = 3$, $P < 0.0001$). Differences were not due to an allele effect on grain volume weight. The cause of generally lower yield in previous evaluations of the $wx^b$ inbred lines evaluated in Yerka et al. (2015a) is not known; however, grain yield was highest for hybrids with the $wx^b$ Wheatland parent in the current study. The above observations suggest that the *Wx* locus may be linked to alleles affecting height and yield; however, the lack of a consistent positive or negative association between *wx* alleles, height, and yield in Yerka et al. (2015a) suggests that the three traits are not tightly linked. Further research will be needed to definitively test additive, dominance, and epistasis variance in a broader set of *wx* hybrids with different combinations of $wx^a$ and $wx^b$ alleles to identify favorable haplotypes.

Jampala et al. (2012) evaluated the effect of WT and *waxy* endosperm on grain yield and yield components in 100 $F_{2:4}$ derived recombinant inbred lines from a cross between RTx2907 (*wx*) and P850029, a WT line with highly digestible protein (HD). Analysis of variance of progeny segregating for WT, *wx*, HD, and stacked traits demonstrated no significant effect of endosperm type on 100-kernel weight. Entry within endosperm type had a significant ($P \leq 0.05$) effect on grain yield and a highly significant ($P \leq 0.01$) effect on height, although overall *wx* grain yield did not differ from that of the WT (Jampala et al., 2012). Jampala et al. (2012) concluded that genetic linkage resulting in reduced yields of *wx* sorghum may be unique to the population and parents tested (e.g., background effects). Observed differences in grain yield and height among near-isogenic WT and *wx* lines by Yerka et al. (2015a,b), and hybrids in the current study, support this conclusion.

## Grain Composition

Grain moisture and crude protein did not differ among hybrids, but differences were observed for other measures of grain composition (Table 3). Crude fiber was lower ($n = 2$, $P = 0.0043$) for $wx^b$ × $wx^a$ (1.67 g $kg^{-1}$) than for WT × WT (1.82 g $kg^{-1}$). Fat, ash, starch, and calculated

**Table 3. Grain composition of near-isogenic wild-type (WT), *waxy*, and hetero*waxy* Wheatland × Tx430 sorghum hybrids. Data were pooled over four environments: Mead and Lincoln, NE, in 2009 and 2010.**

| Hybrid entry† | Moisture | FAN‡ | CP | CF | Fat | Ash | Starch | TDN |
|---|---|---|---|---|---|---|---|---|
| | g kg$^{-1}$ | mg L$^{-1}$ | g kg$^{-1}$ dry matter | | | | | |
| WT × WT | 16.38a§ | 54.39a | 9.75a | 1.82c | 3.57a | 1.77a | 70.62a | 81.31a |
| WT × *wx*$^a$ | 16.27a | 49.94a | 9.66a | 1.75bc | 3.66ab | 1.74a | 70.83a | 81.41ab |
| *wx*$^b$ × *wx*$^a$ | 16.08a | 63.86b | 9.80a | 1.67ab | 3.91c | 1.84b | 72.06b | 81.55b |
| *wx*$^b$ × WT | 16.26a | 50.06a | 9.83a | 1.65a | 3.75b | 1.75a | 71.00a | 81.55b |
| *P*-value¶ | 0.0599 | <0.0001 | 0.9175 | 0.0041 | <0.0001 | 0.0122 | 0.0196 | 0.0023 |

† *wx*$^a$ and *wx*$^b$ denote two recessive alleles conferring the *waxy* phenotype.

‡ FAN, free amino N; CP, crude protein; CF, crude fiber; TDN, calculated total digestible nutrients.

§ Means followed by the same letter within columns do not differ at $P = 0.05$.

¶ ANOVA *F*-test for the overall effect of hybrid.

TDN of *wx*$^b$ × *wx*$^a$ (3.91, 1.84, 72.06, and 81.55 g kg$^{-1}$, respectively) were higher than that of WT × WT (3.57, 1.77, 70.62, and 81.31 g kg$^{-1}$, respectively). The hetero-*waxy* hybrids were similar to each other for all characters except crude fiber ($n = 2$, $P = 0.0528$), for which WT × *wx*$^a$ (1.75 g kg$^{-1}$) was similar to WT × WT ($n = 2$, $P = 0.1438$) and *wx*$^b$ × WT (1.65 g kg$^{-1}$) was similar to *wx*$^b$ × *wx*$^a$ ($n = 2$, $P = 0.6235$). Fat content of hetero*waxy* grain was intermediate (3.66–3.75 g kg$^{-1}$) between *wx*$^b$ × *wx*$^a$ and WT × WT ($n = 4$, $P < 0.0001$).

Observed WT, *wx*, and hetero*waxy* hybrid grain composition profiles are numerically similar to results reported by Wu et al. (2010), although the ranking of individual WT or *wx* characters differed. This was likely due to environmental variation and the specific genetic backgrounds tested. A study of 70 grain sorghums differing at *Wx* showed that only fat content and protein digestibility significantly differed (they were higher) in *wx* lines compared with WT lines (Wu et al., 2007). The higher fat content of *wx* sorghum lines and hybrids is consistent with observations that the WT *Wx-D1* allele of wheat reduced grain lipid content (Yasui 2012) and the WT *Wx* allele of rice decreased fat and protein content (Yu et al., 2009).

In previous studies, starch content of the grain had a positive effect on ethanol yield (Wu et al., 2007, 2010; Yan et al., 2011), while protein, crude fiber, tannins, and ash had negative effects (Wu et al., 2007). The crude fiber, starch, and total energy values observed for the four hybrids agree with results of Wu et al. (2013), who also reported that average ethanol fermentation efficiency was highest for *wx*$^b$ × *wx*$^a$ (90.6%), intermediate for *wx*$^b$ × WT (90.1%) and WT × *wx*$^a$ (90.2%), and lowest for WT × WT (89.9%) ($n = 4$, $P = 0.0375$).

Free amino-N content impacts ethanol fermentation efficiency because yeast is unable to utilize intact proteins and depends on free amino groups and short peptides as a N source. This parameter is especially important during the first 30 h (Yan et al., 2011), and greatly influences fermentation efficiency (Wu et al., 2010). Protein hydrolysis results in multiple small peptide fragments, and thereby, increases FAN content (Wu et al., 2010; Yan et al., 2011). In the present study, FAN content of *wx*$^b$ × *wx*$^a$ (63.86 mg mL$^{-1}$) was highest ($n = 4$, $P < 0.0001$), whereas that of the WT × WT, *wx*$^b$ × *Wx*, and *Wx* × *wx*$^a$ ranged from 49.94 to 54.39 mg mL$^{-1}$ and did not differ. The range of FAN content observed in these hybrids was comparable to that observed among WT, *waxy*, and hetero*waxy* sorghum lines evaluated by Wu et al. (2010) and Cremer et al. (2014), and was toward the lower end of the range of *waxy* lines evaluated by Yan et al. (2011). The FAN content of finished sorghum mash in ethanol production should be 150 to 300 mg mL$^{-1}$ (similar to maize mashes) to complete starch fermentation into ethanol in 72 h (Wu et al., 2010). Grain with higher FAN content should reduce the amount of supplemental N needed and, therefore, the cost of production.

Representative thermograms from DSC analysis of hybrid grain starch are presented in Fig. 1. Amylopectin and amylose gelatinizatized at approximately 75 and 106°C, respectively (Fig. 1A); and the amylose–LPC complex formed at approximately 91°C (Fig. 1B) for all hybrids. The least-squares means of peak onset, maximum and end temperatures, peak enthalpy of transition, and calculated amylose content from DSC are in Table 4. Positive enthalpies denote endothermic reactions, whereas negative enthalpies denote exothermic reactions. Based on enthalpy of amylose–LPC complex formation, calculated amylose content was highest for WT × WT (34.80 g kg$^{-1}$) starch, lowest for *wx*$^b$ × *wx*$^a$ (7.66 g kg$^{-1}$) starch, and intermediate for *wx*$^b$ × *Wx* (25.06 g kg$^{-1}$) and *Wx* × *wx*$^a$ (27.20 g kg$^{-1}$) starches ($n = 4$, $P < 0.0001$). These data are similar to previously reported sorghum grain amylose contents (Rooney and Serna-Saldivar 2000; Zhu 2014), although amylose contents of the *wx*$^b$ × *wx*$^a$ and WT × WT hybrid starches were slightly higher than the expected 0 to 5 and 20 to 30 g kg$^{-1}$ for homozygous *waxy* and WT sorghum starches, respectively. It is unclear whether this observation is due to different methodologies used to quantify amylose (Polaske et al., 2005; Sievert and Holm 1993) or to low levels of GBSS activity in *wx*$^b$ genotypes (25% that of WT, Sattler et al., 2009). When colorimetric (iodine-binding) assays were compared with the DSC assay, DSC provided a more accurate estimate of amylose content in high-amylose

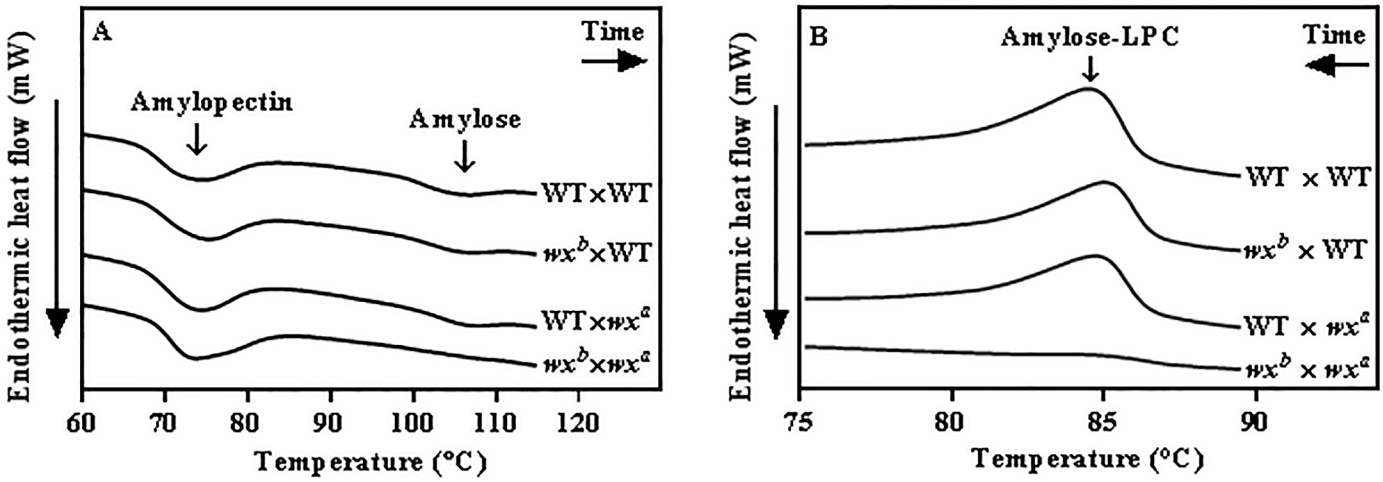


Figure 1. Differential scanning calorimetry (DSC) thermograms from purified grain starch of near-isogenic wild-type (WT), *waxy*, and hetero*waxy* Wheatland × Tx430 hybrids. (A) amylopectin and amylose starch gelatinization during an initial heating phase. (B) melting of the amylose–1-α-lysophosphatidylcholine (LPC) complex during a subsequent cooling phase. Representative thermograms are shown for each hybrid. Hybrid grain samples ($n$ = 4) were composited within locations ($n$ = 2, Mead and Lincoln, NE) over 2 yr (2009 and 2010) and five replicates within each year. Three technical replicates of hybrid starch from both locations ($n$ = 24) were analyzed in three separate DSC runs. The onset, maximum, and endpoint temperatures of each peak, peak area, and enthalpy of transition ($\Delta H$, J g$^{-1}$) were determined with PyrisTM software (Table 4). Enthalpy from formation of the amylose–LPC complex was used to relate to amylose starch content using a wheat starch standard curve.

**Table 4. Differential scanning calorimetry (DSC) analysis and amylose determination of grain starch from near-isogenic wild-type (WT), *waxy*, and hetero*waxy* Wheatland × Tx430 sorghum hybrids. Purified grain starch from each hybrid was pooled over five replicates and 2 yr (2009 and 2010) within each of two environments (Mead and Lincoln, NE). Composite samples were subsampled three times to generate technical replicates for three separate DSC runs.**

| Hybrid entry† | Amylopectin gelatinization | | | | Amylose gelatinization | | | | Amylose–LPC complex | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Onset | Max | End | $\Delta H$‡ | Onset | Max | End | $\Delta H$ | Onset | Max | End | $\Delta H$ | Amylose§ |
| | ——— °C ——— | | | J g$^{-1}$ | ——— °C ——— | | | J g$^{-1}$ | ——— °C ——— | | | J g$^{-1}$ | g kg$^{-1}$ |
| WT × WT | 67.06a | 74.92a | 82.82a | 15.24a | 100.11ab | 105.83a | 113.62a | 4.71c | 93.27a | 91.17a | 86.19a | –7.97c | 34.80c |
| WT × *wx*$^a$ | 67.99a | 74.92a | 82.76a | 15.87a | 99.43a | 106.22a | 114.78a | 3.20b | 93.58a | 91.33a | 86.21a | –5.94b | 25.06b |
| *wx*$^b$ × *wx*$^a$ | 68.42a | 73.43a | 84.23a | 18.67b | 100.99b | 106.46a | 114.11a | 0.26a | 94.62b | 91.81a | 86.07a | –0.77a | 7.66a |
| *wx*$^b$ × WT | 67.89a | 74.82a | 82.91a | 15.87a | 98.88a | 106.08a | 113.23a | 3.54b | 93.16a | 91.26a | 86.12a | –6.45b | 27.20b |
| *P*-value¶ | 0.0657 | 0.4247 | 0.2071 | 0.0391 | 0.0123 | 0.2448 | 0.3500 | 0.0002 | 0.0406 | 0.1733 | 0.9801 | <0.0001 | <0.0001 |

† *wx*$^a$ and *wx*$^b$ denote two recessive alleles conferring the *waxy* phenotype.

‡ $\Delta H$, melting enthalpy of transition.

§ Calculated amylose starch content based on enthalpy on formation of the amylose–1-α-lysophosphatidylcholine (LPC) complex during cooling of gelatinized starch.

¶ ANOVA *F*-test for the overall effect of hybrid.

(>50 g kg$^{-1}$) starches (Polaske et al., 2005; Sievert and Holm 1993) but slightly overestimated amylose content of WT wheat and maize starches (~30 g kg$^{-1}$ amylose) (Sievert and Holm 1993). However, DSC is preferred over colorimetric assays due to the additional information about starch chemistry (e.g., amylopectin properties) it provides. Near-isogenic hybrids homozygous for *wx*$^a$ and *wx*$^b$ will be needed to elucidate whether low GBSS activity increases amylose content, which merits further research.

Amylopectin and amylose gelatinization enthalpies corresponded to observed amylose content: *wx*$^b$ × *wx*$^a$ starch had the greatest amylopectin gelatinization enthalpy (18.70 J g$^{-1}$), while WT × WT starch had the least (15.24 J g$^{-1}$). Similarly, amylose gelatinization enthalpy was least for *wx*$^b$ × *wx*$^a$ (0.26 J g$^{-1}$) and greatest for WT × WT (4.71 J g$^{-1}$) starches. Hetero*waxy* hybrid starches did not differ from each other for any peak parameter. Observed amylopectin gelatinization enthalpy of hetero*waxy* starch was similar to that of WT × WT starch but less than that of *wx*$^b$ × *wx*$^a$ starch, while amylose gelatinization enthalpy of hetero*waxy* starch was intermediate between that of *wx*$^b$ × *wx*$^a$ and WT × WT starches. This latter result is likely due to hetero*waxy* amylose/amylopectin ratio being nearer to that of WT × WT.

A recent evaluation of WT and high-amylose durum wheat starches showed that lines with increased (10–15 g kg$^{-1}$ more, relative to WT) grain amylose content had lower amylopectin gelatinization temperatures and enthalpies (Hogg et al., 2013). The results were attributed to mutation of starch synthase IIa enzyme, which disrupted the biosynthesis of amylopectin. Wild-type amylopectin

exists in an ordered, crystalline form (Vladimir et al., 2004); reduced gelatinization temperature may be diagnostic of its compromised structure (Hogg et al., 2013). The difference in grain amylose content between $wx^b \times wx^a$ and WT × WT sorghum hybrids in the current study was much greater than 15 g $kg^{-1}$, yet no differences were observed in amylopectin or amylose gelatinization maximum temperatures (corresponding to the gelatinization temperature reported by Hogg et al. [2013]). This observation suggests that amylopectin synthesis, and the production of amylose among WT and hetero*waxy* hybrids, did not differ. Differences in enthalpy during amylopectin and amylose gelatinization were due to quantity and not quality of the respective polymers.

Previous research showed that environment can affect sorghum grain amylose content, possibly as a result of changes in the relative proportions of corneous to floury endosperm within the kernel (Ring et al., 1989). However, a recent review of the literature for the effects of environmental stress on starch composition and functionality in cereals suggested that amylose content of sorghum starch may vary less in response to heat stress than starches from maize, wheat, or rice (Beckles and Thitisaksakul 2014). If so, the relative stability of amylose production under increasingly variable climatic conditions would make hetero*waxy* sorghum hybrids strong competitors with other crops in environments where heat and water deficit stress tolerance are needed for the production of starches with customized amylose content.

## CONCLUSION

The agronomic performance and grain yield of four near-isogenic WT, *waxy*, and hetero*waxy* Wheatland × Tx430 sorghum hybrids were evaluated. While *waxy* sorghum has historically been associated with lower grain yield than WT, the *waxy* and hetero*waxy* hybrids in the current study demonstrated equal or superior field emergence, days to 50% anthesis, grain yield, and grain volume weight relative to the WT. The reduced amylose content of *waxy* and hetero*waxy* hybrid grain relative to WT should reduce the energy inputs necessary for efficient fermentation and provide opportunities to customize some food products. These high-yielding sorghum hybrids are suitable for expanded production of reduced-amylose starch into regions where water is limiting.


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